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Identification of medically important pathogenic fungi by reference strand-mediated conformational analysis (RSCA)

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This report describes the application of reference strand-mediated conformational analysis (RSCA), a novel DNA typing technique, for the identification of clinically significant fungal pathogens. RSCA is a heteroduplex-based conformational method which relies on detecting differences in the DNA conformation of heteroduplexes generated in this study by the annealing of different fungal 18S rRNA amplicons to a common fluorescent-labelled reference (FLR). These heteroduplexes are then observed with laser-based instrumentation and computer software to detect differences in the DNA conformation reproducibly. This technique was shown to generate unique and reproducible profiles for the 18S rRNA gene sequences of a number of medically important fungi, distinguishing different *Candida* species (*C. albicans*, *C. kefyr*, *C. dubliniensis*, *C. lusitaniae*, *C. guilliermondii*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. sake* and *C. parapsilosis*), and in some cases detecting single nucleotide differences between 18S rRNA sequences. The RSCA technique was further evaluated with 50 human clinical isolates of *Candida* spp., previously identified by culture techniques, and was shown to identify the isolates correctly. This technique displays enormous potential as an alternative to DNA sequence determination and has the potential to become an automated technique that can be implemented in the routine setting.

Introduction

The last 20 years have seen unprecedented changes in the pattern of fungal infections in man. These infections have assumed more importance because of their increasing incidence in patients with acquired immune deficiency syndrome (AIDS), in transplant recipients, in cancer patients and in other groups of debilitated or immunocompromised individuals [1]. New pathogens have emerged, while others have been almost eradicated. Aspergillosis and candidosis are the most common mycoses in immunocompromised patients [2] and recently *Candida* species have been

reported as the fourth most common cause of blood-stream infection in the USA [3].

Early diagnosis is required to improve the prognosis of the immunocompromised patient with systemic fungal infection [4]. Conventional methods for diagnosis of a fungal infection rely on microscopy, culture and antigen detection, which show low sensitivity or specificity. In an attempt to provide a simple, rapid and reliable method to identify fungal isolates, PCR-based assays have been developed to specifically amplify DNA from fungal pathogens. PCR-based amplification of target DNA offers the potential for a more specific and sensitive alternative to conventional methods for the detection and identification of fungal pathogens. Several techniques have been reported to distinguish different fungi detected by universal PCR primers. These include restriction fragment length polymorphism [5, 6], hybridisation of the amplicon

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with a specific probe [1, 7–10], single-strand conformational polymorphism (SSCP) [11], nucleic acid sequence-based amplification [12] and the LightCycler system [13]. This report describes an alternative approach to these strategies by utilising the technique of reference strand-mediated conformational analysis (RSCA) which, when combined with PCR amplification, allows the detection and specific identification of pathogenic fungi.

RSCA is a heteroduplex-based typing technique which differs from other sequence-based typing methodologies in that the type is assigned on the basis of differences in DNA conformation (due to mismatches) that occur when two different sequences are hybridised to each other (heteroduplex), retarding the migration of the heteroduplex in a non-denaturing polyacrylamide gel [14]. The principal application of RSCA to date has been in the transplantation setting, where it is used to define the highly polymorphic human leucocyte antigen (HLA) genes and select the best match for individuals awaiting solid organ and bone marrow transplantation [15–17]. Within this field, RSCA has established itself as a DNA fingerprinting technique that is unrivalled in its simplicity, speed, specificity, accuracy, reproducibility between gels and discriminatory power (it can detect a single nucleotide difference), and clearly displays considerable potential as a molecular diagnostic tool in the broader clinical setting. This study evaluated the potential of RSCA to recognise and distinguish various fungi based on sequence variation in a small hypervariable region (281 bp) of the 18S rRNA gene. Furthermore, to assess its clinical applicability, a number of *Candida* isolates that had previously been identified by conventional mycological culture techniques were analysed by RSCA.

Materials and methods

Fungal isolates

Candida guilliermondii, *C. kefyr*, *C. krusei*, *C. dubliniensis*, *C. sake*, *Sporothrix schenckii* and *Saccharomyces cerevisiae* reference strains were obtained from the National Collection of Pathogenic Fungi (NCPF nos 3099, 3898, 3953, 3949, 3860, 3287 and 319, respectively; PHLS Mycology Reference Laboratory, Bristol). Other fungal species were obtained from the culture collection held at the Mycology Laboratory, Royal Victoria Hospital, Belfast; these included *Scedosporium apiospermum*, *Sced. prolificans*, *Trichophyton mentagrophytes* var. *granulosum*, *T. mentagrophytes* var. *interdigitale*, *Microsporum persicolor*, *M. gypseum*, *M. canis*, *T. rubrum*, *Acremonium* species, *Alternaria alternata*, *Aspergillus fumigatus* (two different isolates), *C. parapsilosis*, *C. lusitanae*, *C. tropicalis*, *C. albicans*, *C. glabrata*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Rhodotorula mucilogenosa*, *Fusarium* species and *Asp. flavus*. In addition to these, 50 recent *Candida* clinical isolates were also provided by

the Mycology Laboratory but their identity was withheld until after RSCA analysis.

Culture conditions

All NCPF fungal strains were grown from lyophilised pellets on MALT Agar (Becton Dickinson UK) at 37°C for 48–72 h except for *C. sake* which was grown at 28°C for 48 h. All the other fungal species, including the 50 clinical yeast isolates, were obtained on agar plates from RVH, Belfast. All the fungal strains and clinical isolates employed in this study were identified by standard mycological methods of germ-tube formation in serum or carbohydrate assimilation tests with the API 20C kit (bioMérieux, Hazelwood, MO, USA).

DNA extraction

DNA was extracted from each of the reference fungal strains and yeast isolates employed in this study by a modification of a previously described method [5, 18]. Briefly, two loops of fungal biomass (yeast or mycelia) from several day-old cultures on Sabouraud's Dextrose Agar (Becton Dickinson) were transferred to 2-ml sterile polypropylene screw-top tubes with o-ring seals (Sarstedt, Newton, NC, USA) and resuspended in 400 µl of extraction buffer – Triton X-100 2%, SDS 1%, 80 mM NaCl, 50 mM Tris-Cl (pH 8.0), 5 mM EDTA. Lysis was achieved as described previously [5] and the resulting fungal DNA pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8) at a concentration of 100 ng/µl and stored at –20°C until required.

PCR

PCR was performed under stringent conditions in a hospital diagnostic laboratory. Pre- and post-PCR manipulations were performed in separate designated rooms with separate positive-displacement pipette devices to avoid contamination of the samples with foreign DNA. Aerosol-resistant pipette tips were used to handle all liquids. UV light was used to irradiate all equipment used in the pre-amplification steps to prevent contaminating DNA from causing false-positive results.

The 18S rRNA hypervariable region from each of the fungal DNA extracts was amplified by PCR with universal fungal primers Fn1f (ATT GGA GGG CAA GTC TGG KG), based on Einsele *et al.* [7], and Fn1r (GTC CTA TTC YAT TAT YMC ATG CTA A) which correspond to positions 543–562 and 802–778 in the *C. albicans* 18S rRNA gene sequence (GenBank accession no. M60302), respectively, amplifying a maximum fragment of 281 bp. PCR was performed in a 100-µl volume containing 10 mM Tris-HCl, (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 200 µM of dNTPs, 20 pmoles of each primer, and 2.5 U *Taq* polymerase (PE-Applied Biosystems) with 1 µl of the fungal DNA

added according to standard procedures. PCR was performed with an initial denaturation cycle of 96°C for 5 min followed by 30 cycles of 1 min at 96°C, 30 s at 55°C and 1 min at 72°C and extension at 72°C for 5 min followed by 15°C 'forever'.

DNA sequencing

To confirm the identity of each of the fungi used in this study, semi-automated fluorescent sequencing with an ABI 373A sequencer (ABI, Foster City, CA, USA) was performed as described previously [19] with the same primers as above. The sequences obtained were screened against the EMBL and GenBank databanks (National Centre for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/BLAST/>) and sequence alignments were performed with the MAGI multiple sequence alignment at the UK HGMP Resource Centre (<http://www.hgmp.mrc.ac.uk/>) against the reference (*Asp. niger*) (see Fig. 1).

Preparation of fluorescent-labelled references (FLRs)

Fluorescent-labelled references (FLRs) were prepared from DNA extracted from *Asp. niger*, *Sced. prolificans* and *Spor. schenckii* by PCR as described above except that the 5' primer (Fn1f) was labelled with the fluorochrome IRD800 (MWG, BIOTECH, Milton Keynes). The FLR was purified by the QIAquick PCR purification kit as outlined in the manufacturer's protocol (Qiagen, West Sussex).

Duplex formation

Duplexes were formed as described previously [16]. Briefly, 1 µl of FLR PCR product was mixed with 3 µl of sample PCR product, derived from the wide range of fungal pathogens included in this study. The samples were then denatured at 96°C for 4 min to separate the sense and antisense strands of the DNA. The temperature was then reduced over a period of 5 min to 55°C and held there for 1 min, and then reduced over a period of 5 min to 15°C and held there for 8 min, to facilitate hybridisation of the complementary sense and antisense strands. Ficoll loading buffer (4 µl: Ficoll 15%, bromophenol blue 0.25%) was added to the samples before electrophoresis.

Electrophoresis

Duplexes were separated with a LI-COR 4200 automated sequencer (MWG, BIOTECH). One µl of each duplex sample was loaded on to a non-denaturing polyacrylamide 5.5% gel (Long Ranger™ Gel solution; Flowgen) and electrophoresis was performed with 35 W constant power and the temperature maintained at 40°C. Gels were 66 cm long and 0.25 mm thick. Each FLR duplex combination was visualised and

analysed after electrophoresis with RFLP scan software (Scanalytics, Fairfax, USA).

Results

Sequence analysis and PCR amplification

The PCR products generated with the broad range primers for the fungal species used in this study were sequenced and their identities were confirmed by BLAST searches (EMBL/GenBank database). Fig. 1 shows all the sequences of the fungal species aligned for the 281-bp PCR product against the sequence of *Asp. niger*.

RSCA analysis

RSCA is a technique that involves detecting differences (mismatches) in the heteroduplexes generated between the FLR strand and the complementary strand of the sample PCR product. Fig. 2 is a schematic diagram detailing the mechanism behind this technique. Essentially a FLR PCR product is generated for the sequence of interest. The fluorescent label is restricted to one of the DNA strands of the FLR PCR product as only one of the two PCR primers contains a 5' fluorescent label. The FLR is then mixed with the PCR product for the same region (unlabelled) from a different sample or species to be tested. A process of denaturation followed by annealing is allowed to take place to facilitate hybridisation of the complementary sense and antisense strands. Annealing can also occur between the sense and antisense strands of the different DNA species present in the mixture, forming heteroduplexes (Fig. 2). The duplexes formed are separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) with an automated sequencer and only those duplexes possessing a fluorescent label are detected by the laser. Nucleotide mismatches cause loopouts in the heteroduplex DNA structure retarding the migration of the heteroduplex, relative to the homoduplex, producing highly reproducible migration patterns (RSCA profiles).

To evaluate the potential of RSCA to recognise and distinguish various fungi based on sequence variation in the 18S rRNA gene, the 18S rRNA amplicons derived from the 28 different fungal species were first independently subjected to RSCA analysis with three different FLRs, namely *Asp. niger*, *Sced. prolificans* and *Spor. schenckii*. While each of the three FLRs assessed in the initial RSCA analysis screen generated a diverse and different array of RSCA profiles (data not shown), the majority of which were distinct for each of the fungal genera examined, the FLR derived from *Asp. niger* resulted in a greater number of distinct RSCA profiles for the 28 different fungal species studied which were easily resolved because of their disperse nature across the expanse of the gel image (Fig. 3). The homoduplex FLR migrates at the bottom of the gel, the

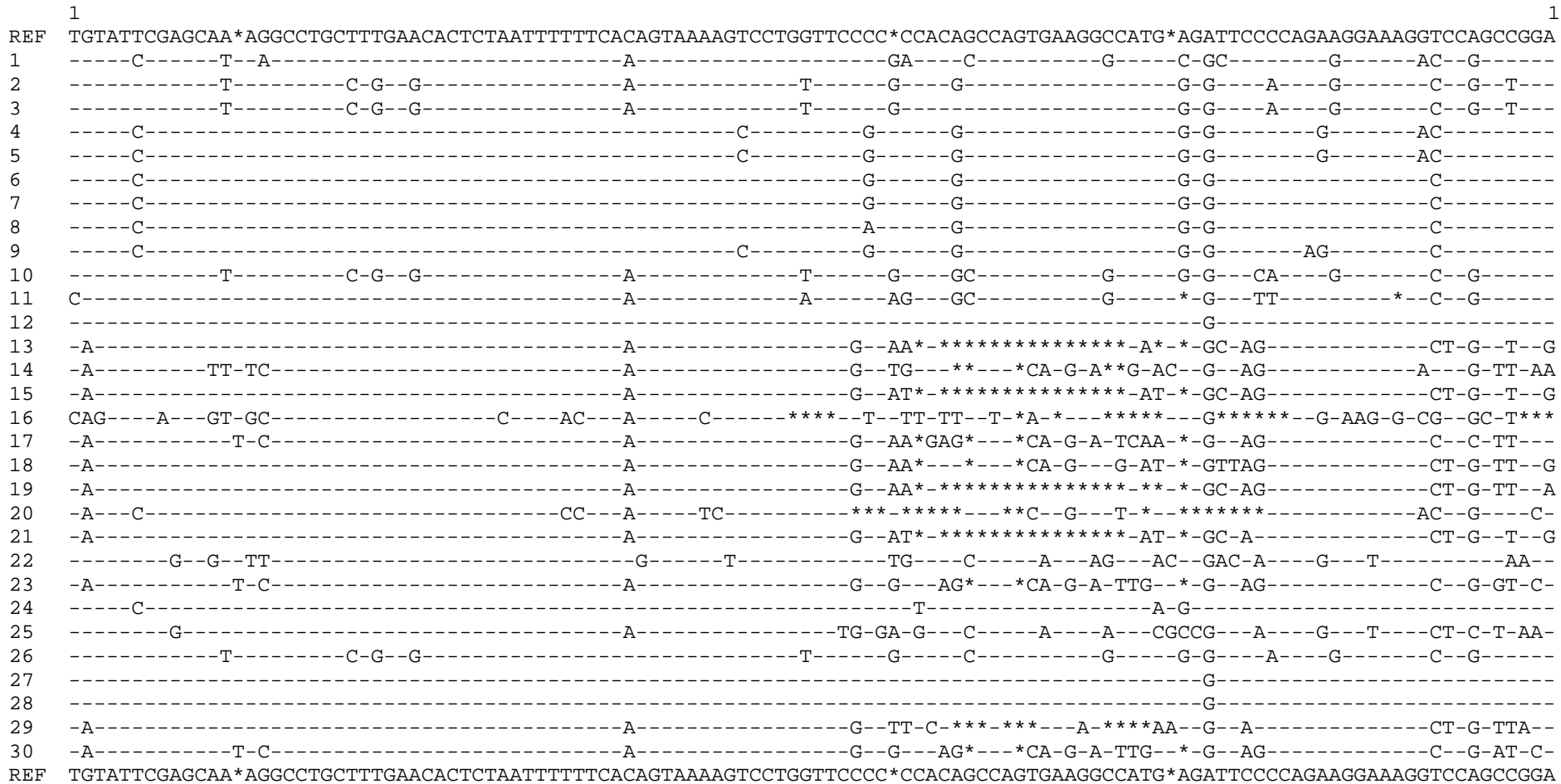


Fig. 1. Alignment of the DNA sequences of the fungal species. The alignment shows the sequences for the 281-bp fragment generated with the primers Fn1f and Fn2r (primer sequences are not shown in the alignment). REF refers to the sequence of *Asp. niger* FLR; *, denotes no base present at that position; -, denotes that the sequence is the same for that species. Numbers 1–30 refer to the following organisms: 1, *Spor. schenckii*; 2, *Sced. apiospermum*; 3, *Sced. prolificans*; 4, *T. mentagrophytes* var. *granulosum*; 5, *T. mentagrophytes* var. *interdigitale*; 6, *M. persicolor*; 7, *M. gypseum*; 8, *M. canis*; 9, *T. rubrum*; 10, *Acremonium* spp.; 11, *Alt. alternata*; 12, *Asp. fumigatus*; 13, *C. parapsilosis*; 14, *C. kefyi*; 15, *C. dubliniensis*; 16, *C. lusitaniae*; 17, *Sacch. cerevisiae*; 18, *C. guilliermondii*; 19, *C. tropicalis*; 20, *C. krusei*; 21, *C. albicans*; 22, *Crypt. neoformans*; 23, *C. glabrata*; 24, *Cocc. immitis*; 25, *R. mucilogenosa*; 26, *Fusarium* sp.; 27, *Asp. fumigatus* (second isolate); 28, *Asp. flavus*; 29, *C. sake*; 30, *C. glabrata* (clinical isolate displaying distinct RSCA profile).

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REF ***CCAGTACTCGCGGTG**AGGCGGACCGGCCAGCCAGACCCAAGGTTCAACTACGAGCTTTTTAACTGCAACAACCTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGG

1 -----G-A-----G-----

2 ---T---G-A-C-----G-----G-----CA-----

3 ---T---G-A-C-----G-----G-----CA-----

4 -----G-A--C-----G-----

5 -----G-A--C-----G-----

6 -----G-A--C-----G-----

7 -----G-A--C-----G-----

8 -----G-A--C-----G-----

9 -----G-A--C-----A-----G-----

10 -----G-A-----G-----G-----CA-----

11 ---G--G-A-----C-----G-----T-----

12 -----

13 --T-----G-ATCAAAAA--AT-----AG-----T-----

14 --A---G-G--ACA-*AA-AT-----A-----A-----C-----

15 --T-----G-ATCA*AAA--AT-----AG-----T-----

16 -----G---CT**--A-----AC-G-GCCT-----C-----

17 AAT-----A--***AAAA-AT-----A-G-G--A-----

18 --T-----CA*AAA-----A-AG--A-----

19 --T-----G-ATCAGAAA*-AT-----A-AG-----T-----

20 --A---G--TA-CA-*---TA---T-GC--G---A-----

21 --T-----G-ATCA*AAA--AT-----AG-----T-----

22 --*---G-A-T-C-----A-----C-TC-----TG-A--G-----C-----

23 T-T-----GA--***AAAA-AT-----C--G---A-----

24 -----G-A--C-C--A-----

25 ---A---A-A-CAAG-A-----TGA-AGAG--G-A--G-----G-----T-----

26 -----A-----G-----A-----CA-----

27 -----

28 -----

29 --T-----A-*CAAA-----T-A--AG-----T-----

30 T-T-----GA--***AAAA-AT-----C--G---A-----

REF ***CCAGTACTCGCGGTG**AGGCGGACCGGCCAGCCAGACCCAAGGTTCAACTACGAGCTTTTTAACTGCAACAACCTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGG

Fig. 1. (continued)

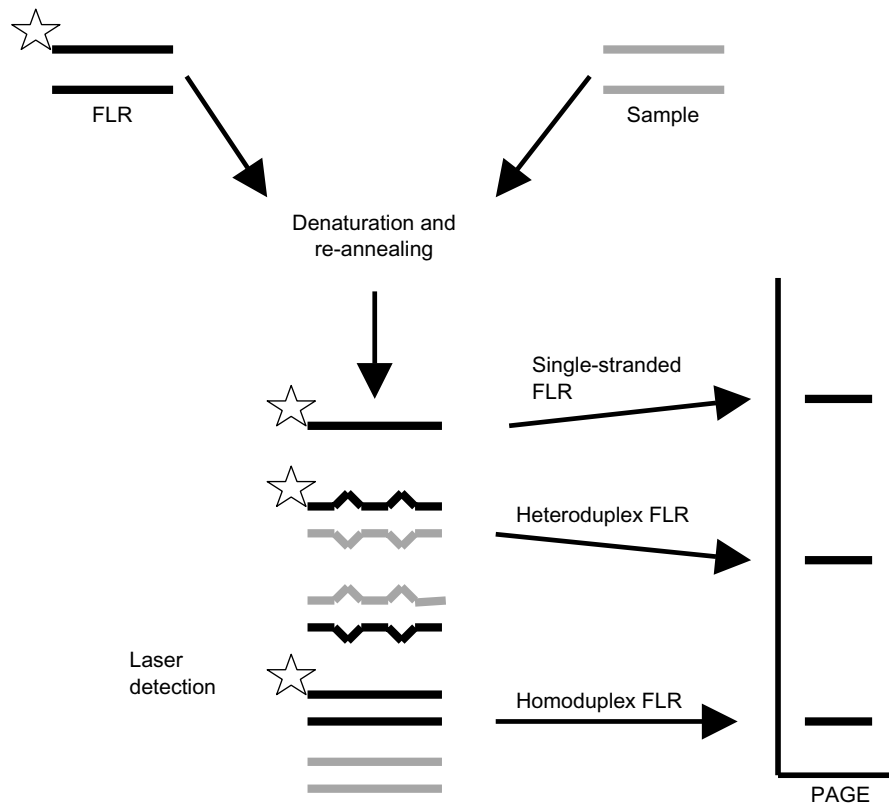


Fig. 2. Diagrammatic representation of the RSCA method. The FLR PCR product contains a fluorescent label on its sense strand shown by the star. The FLR is mixed with the PCR product of 18S rRNA gene region and the sense and antisense strands of the DNA amplicons are denatured. The strands are then allowed to re-anneal generating homoduplex, heteroduplex and single-stranded FLR. The duplexes formed are then separated by PAGE and only those duplexes containing the FLR strand are detected with the laser. In this diagram two mismatches are observed in the heteroduplex which causes changes in the tertiary structure of the DNA strands, causing the migration profile to be different from that of the homoduplex FLR.

labelled single-stranded FLR migrates at the top, and in between this region the different labelled heteroduplexes migrate giving the various unique RSCA profiles for each fungal species tested.

Unique migration patterns, i.e., RSCA profiles, were obtained for each member of the *Candida* genus examined with the *Asp. niger* FLR (Fig. 3, lanes 13–16, 18–21, 23 and 29), namely *C. parapsilosis*, *C. kefyr*, *C. dubliniensis*, *C. lusitanae*, *C. guilliermondii*, *C. tropicalis*, *C. krusei*, *C. albicans*, *C. glabrata* and *C. sake*, permitting consistent differentiation between these particular organisms. Interestingly, this sequence analysis revealed only a single nucleotide difference between *C. albicans* and *C. dubliniensis* NCPF 3949 in the region of the 18S rRNA gene examined in this study. Therefore, the distinct RSCA profiles obtained for these two organisms (Fig. 3, lanes 15 and 21) highlight the discriminatory power of RSCA analysis. However, it should be pointed out that the nucleotide sequence of *C. dubliniensis* reported here was different from that previously documented in the EMBL/GenBank databank (accession no. X99399). In the present study, a G nucleotide was observed at nucleotide positions 103 and 125 (Fig. 1) in the sequence data for *C. dubliniensis*, whereas the docu-

mented *C. dubliniensis* sequence does not contain these two nucleotides (deletions).

A number of other medically important fungi such as *Sacch. cerevisiae* (Fig. 3, lane 17), *Crypt. neoformans* (Fig. 3, lane 22) and *R. mucilogenosa* (Fig. 3, lane 25) also displayed unique RSCA profiles, easily discriminated from the rest of the profiles observed. Furthermore, distinct profiles were obtained for *T. rubrum*, *Acremonium* sp. and *Alt. alternata* (Fig. 3, lanes 9–11, respectively).

The RSCA profiles obtained for *Sced. apiospermum*, *Sced. prolificans* and *Cocc. immitis* were very similar when *Asp. niger* was employed as the FLR (Fig. 3, lanes 2, 3 and 24, respectively), yet different from all the other profiles observed. While *Sced. apiospermum* and *Sced. prolificans* only differed at a single nucleotide position within the 18S rRNA target region (Fig. 1), which may well be insufficient to significantly alter the conformation of the heteroduplex formed with the *Asp. niger* FLR (22 and 23 mismatches, respectively) and permit resolution, the sequence of *Cocc. immitis* compared with these two organisms was significantly different (26 and 25 differences in total between their sequences) yet generated a heteroduplex

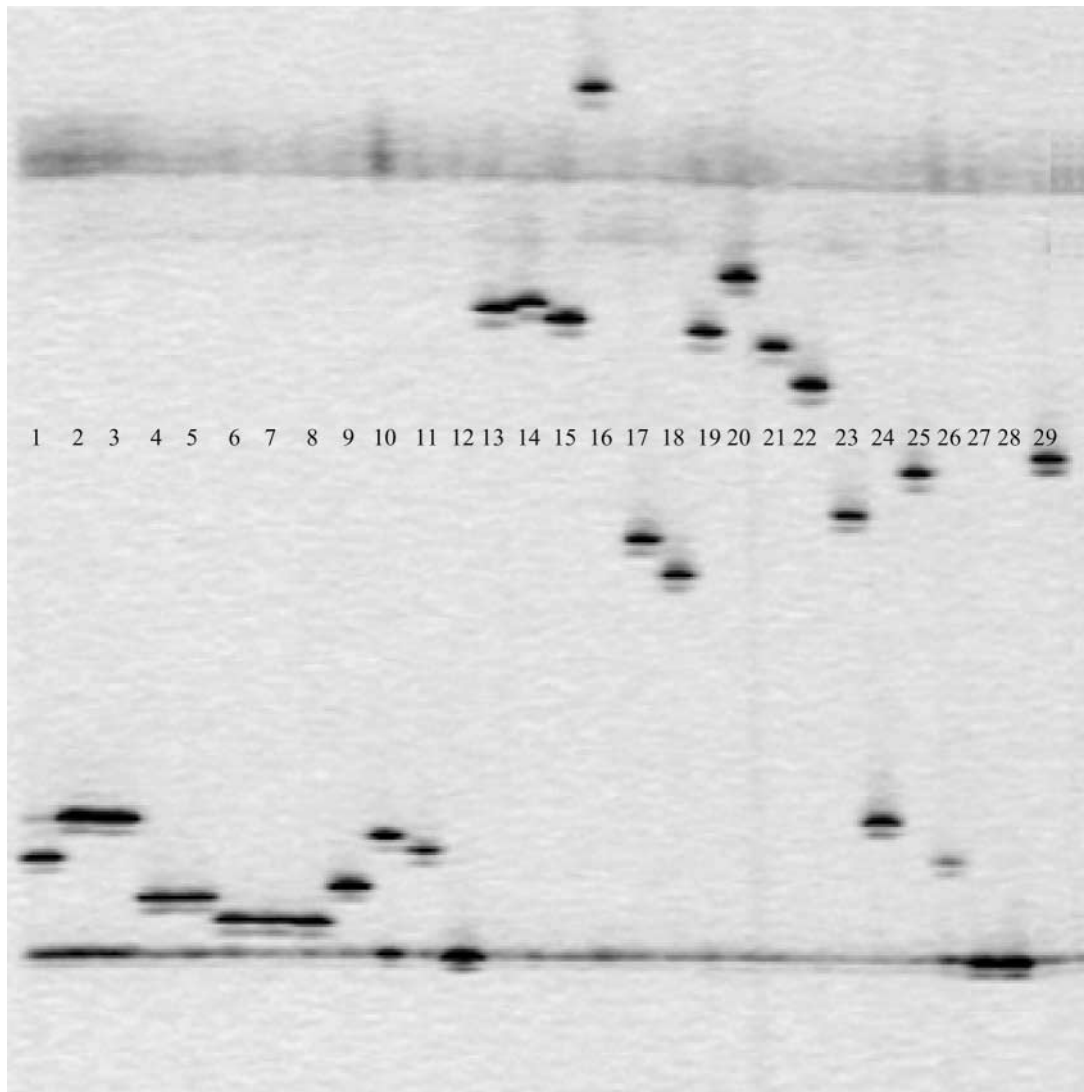


Fig. 3. The RSCA profiles generated between the FLR (*Asp. niger*) and the 18S rRNA PCR products for the various fungal species tested. Lane 1, *Spor. schenckii*; 2, *Sced. apiospermum*; 3, *Sced. prolificans*; 4, *T. mentagrophytes* var. *granulosum*; 5, *T. mentagrophytes* var. *interdigitale*; 6, *M. persicolor*; 7, *M. gypseum*; 8, *M. canis*; 9, *T. rubrum*; 10, *Acremonium* sp.; 11, *Alt. alternata*; 12, *Asp. fumigatus*; 13, *C. parapsilosis*; 14, *C. kefyi*; 15, *C. dubliniensis*; 16, *C. lusitaniae*; 17, *Sacch. cerevisiae*; 18, *C. guilliermondii*; 19, *C. tropicalis*; 20, *C. krusei*; 21, *C. albicans*; 22, *Crypt. neoformans*; 23, *C. glabrata*; 24, *Cocc. immitis*; 25, *R. mucilogenosa*; 26, *Fusarium* sp.; 27, *Asp. fumigatus*; 28, *Asp. flavus*; 29, *C. sake*.

DNA conformation which was retarded to the same extent as the other two related organisms. However, this ambiguity was resolved when *Spor. schenckii* was employed as the FLR, because a distinct and unique profile was obtained for *Cocc. immitis* compared with the identical profiles again obtained for *Sced. apiospermum* and *Sced. prolificans*. A similar scenario of co-migration was also observed for *Spor. schenckii* (Fig. 3, lane 1) and the *Fusarium* sp. (Fig. 3, lane 26), which differed in sequence from each other at 13 nucleotide positions (Fig. 1), but were again resolved when *Sced. prolificans* was employed as the FLR, producing unique and distinct RSCA profiles (data not shown).

All the fungal organisms that had identical nucleotide sequences in the region examined (*T. mentagrophytes*

var *granulosum* and *T. mentagrophytes* var *interdigitale*; *M. persicolor* and *M. gypseum*) also, as expected, produced identical RSCA profiles (Fig. 3, lanes 4 and 5; lanes 6 and 7), highlighting the reproducibility of the RSCA technique between lanes and different DNA and PCR samples. The RSCA profile obtained for *M. canis* (Fig. 3, lane 8) was also identical to the profile obtained for the other two *Microsporum* species (Fig. 3, lanes 6 and 7), despite the presence of a single nucleotide difference (G–A transition at position 64, Fig. 1) separating their respective 18S rRNA gene sequences. As this particular difference in the *M. canis* sequence occurred at one of the 10 existing nucleotide mismatches with the *Asp. niger* reference, it is not surprising that this subtle change (both purines), which is mismatched with a C nucleotide in the reference (Fig. 1), failed to significantly alter the heteroduplex

DNA conformation at this position and result in a different migration value. The other two FLRs employed in this study also failed to distinguish *M. canis* from *M. persicolor* and *M. gypseum*, producing identical RSCA profiles for the three organisms. The profiles obtained for *Asp. fumigatus* (Fig. 3, lanes 12 and 27) and *Asp. flavus* (Fig. 3, lane 28) migrated at the same distance as the double-stranded homoduplex FLR (*Asp. niger*) although there is one nucleotide difference between these *Aspergillus* species and *Asp. niger* (Fig. 1). However, distinct but identical RSCA profiles were obtained for the three *Aspergillus* species when the other two FLRs were employed (data not shown).

It is important to highlight the fact that numerous gels were run under the same conditions and the RSCA profiles generated for each of the fungal organisms tested were found to be highly reproducible, migrating at the same distance relative to the single-stranded FLR and the homoduplex FLR for each gel. This reproducibility was also assessed and confirmed by determining the mobility values (mol. wts) for each RSCA

profile after either assigning arbitrary values of 1000 to the single-stranded FLR and 1 to the homoduplex FLR present in each lane or on occasions including known mol. wt markers on each gel, with the Scanalytics software package of the LI-COR sequencer (data not shown).

Analysis of clinical isolates

The potential of RSCA to identify fungal isolates was assessed with a collection of clinical *Candida* isolates from diverse specimen types obtained from the Mycology laboratory, RVH, and previously identified by conventional methods. The identity of these organisms was withheld until after RSCA analysis was performed on the 281-bp PCR products amplified from the chromosomal DNA of these isolates with *Asp. niger* as the FLR; they included *C. albicans* (30 isolates), *C. glabrata* (9), *C. parapsilosis* (6), *C. tropicalis* (4) and *C. krusei* (1). RSCA correctly identified 46 of these 50 clinical isolates, including all the *C. albicans* (30), *C. parapsilosis* (6), *C. tropicalis* (4) and the single *C. krusei* isolate. A representative gel displaying the

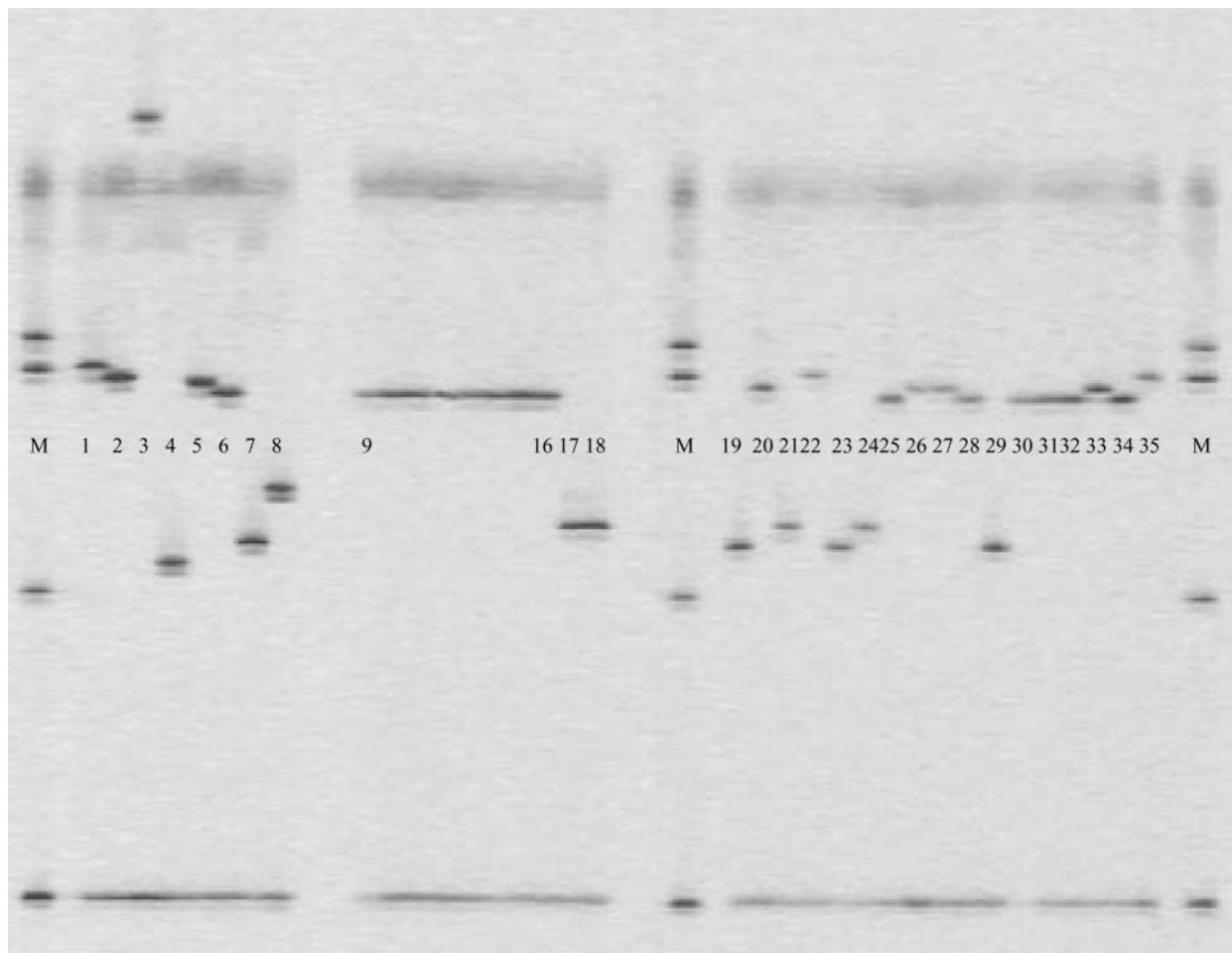


Fig. 4. The RSCA profiles generated between the FLR (*Asp. niger*) and the 18S rRNA PCR products for 27 clinical yeast isolates. Lane M, RSCA profiles for *C. krusei*, *C. parapsilosis* and *C. guilliermondii*. Lane 1, *C. kefyr*; 2, *C. dubliniensis*; 3, *C. lusitaniae*; 4, *Sacch. cerevisiae*; 5, *C. tropicalis*; 6, *C. albicans*; 7, *C. glabrata*; 8, *C. sake*; 9–16, 25, 28, 30–32, 34, *C. albicans* (clinical isolates); 17, 18, 19, 21, 23, 24, 29, *C. glabrata* (clinical isolates); 20, 26, 27, 33, *C. tropicalis* (clinical isolates); 22, 35, *C. parapsilosis* (clinical isolates).

RSCA profiles generated for 27 of these isolates is shown in Fig. 4. The reproducibility of the technique for each type of species is clearly demonstrated in Fig. 4 for *C. albicans* (lanes 9–16, 25, 28, 30–32 and 34), *C. tropicalis* (lanes 20, 26, 27 and 33) and *C. parapsilosis* (lanes 22 and 35). Interestingly, in the case of the nine *C. glabrata* isolates, five were immediately identified as *C. glabrata* and three representatives are shown in Fig. 4 (lanes 19, 23 and 29). The other four *C. glabrata* isolates generated an identical RSCA profile (Fig. 4, lanes 17, 18, 21 and 24) distinct from the known *C. glabrata* profile (lane 7) and all the other RSCA profiles identified to date with the *Asp. niger* FLR. Nucleotide sequence analysis of the 281-bp PCR target region derived from these four isolates revealed that their sequences were identical to each other but contained a single nucleotide difference when compared with the *C. glabrata* sequence (Fig. 1). When a larger region (890 bp) of the 18S rRNA gene sequence encompassing the 281-bp target region was amplified from all four isolates, with primers F3f (GGA ATG AGT ACA ATT TAA ATC YCK TAA CG) and F11r (TAT AAG GGC ATC ACA GAC CTG TTA TTG CCK C), and the nucleotide sequences were determined (again identical to each other), the closest match in the EMBL/GenBank database to this sequence was with *Kluyveromyces delphensis* (accession no. X83823) containing just two mismatches (one of which is the nucleotide difference distinguishing it from *C. glabrata*, Fig. 1), followed closely by *C. glabrata* (accession no. X51831) with five mismatches.

Discussion

This study demonstrated the application of RSCA to the analysis of PCR amplicons generated from the 18S rRNA genes of medically important opportunist fungi. With this technique it was possible to recognise and distinguish different fungal species, including all the *Candida* species examined, within the targeted 281-bp region. All three FLRs used in this study generated RSCA profiles for the fungal species tested (data not shown) but better migration distances/RSCA profiles were observed when *Asp. niger* was used as the FLR. It was also observed that some species generated more retarded RSCA profiles than others, possibly due to the fact that their sequences have more nucleotide mismatches to the reference sequence (Fig. 1). Evidence supporting this is the highly retarded RSCA profile generated for *C. lusitaniae* (Fig. 3, lane 16), which also displayed the greatest number of mismatches (64) with the FLR.

While all the fungal species displaying identical sequences within the target region gave identical RSCA profiles, a number of organisms with distinct 18S rRNA sequences generated RSCA profiles identical to existing ones when *Asp. niger* was employed as the FLR, i.e., *Fusarium* sp. with *Spor. schenckii*, which differ at 15 nucleotide positions, and *Cocc.*

immitis with *Sced. apiospermum* and *Sced. prolificans*, which differ at 23 and 22 nucleotide positions, respectively. Therefore, it seems likely that in these particular instances, the DNA conformation formed in their respective heteroduplexes must be retarded to the same extent despite their predicted structural differences. However, this co-migration ambiguity was resolved by switching the FLR to either *Sced. prolificans* or *Spor. schenckii*, creating different mismatches in an entirely new context and as a result resolving the two different DNA conformations into two distinct RSCA profiles.

RSCA proved somewhat variable when it came to distinguishing single nucleotide differences. Whereas single nucleotide differences between *C. albicans* and *C. dubliniensis*, and *C. glabrata* and the four clinical isolates of *C. glabrata* could be resolved by RSCA analysis, a number of others failed to produce distinct RSCA profiles. In the case of *Asp. fumigatus* and *Asp. flavus* it may relate to the fact that a single nucleotide difference (Fig. 1, position 91) with the reference FLR (*Asp. niger*) is not always sufficient to produce a significant conformational change in the heteroduplex and will be dependent on the nature and position of the mismatch. Although the RSCA profiles for *Asp. fumigatus* and *Asp. flavus* co-migrate with the *Asp. niger* homoduplex FLR, increasing its intensity, identification to the genus level for *Aspergillus* can still be made by virtue of the absence of an RSCA profile anywhere else in the lane. However, distinct yet identical profiles were generated for the *Aspergillus* species by employing the other two references (data not shown). On the other hand, the inability of RSCA to resolve the single nucleotide differences between *M. canis* and *M. persicolor*/*M. gypseum*, *Sced. apiospermum* and *Sced. prolificans*, may relate to the position and context of the difference in relation to the other mismatches (including their number) occurring in the heteroduplex. In some cases the impact of the differentiating nucleotide mismatch may be masked by other neighbouring mismatches and be unable to impose any significant structural change in the overall DNA conformation facilitating resolution, while in other instances a structural contribution will be imposed, producing distinct RSCA profiles.

The observed RSCA profiles were found to be highly reproducible between lanes and also between gels (data not shown). With the exception of four clinical isolates (Fig. 4 lanes 17, 18, 21 and 24), identical migration values/profiles to those anticipated for the clinical yeast isolates were observed when determined with respect to the single-stranded FLR and homoduplex FLR, permitting their immediate identification. The four isolates had been identified routinely as *C. glabrata* by conventional mycological culture techniques, but the 18S nucleotide sequencing analysis suggested that they may have been misidentified and represent *K. delphensis*. *C. glabrata* and *K. delphensis* are known to share a

close phylogenetic relationship through sequence analysis of their mitochondrial cytochrome-c oxidase genes [20]. Indeed, the 18S rRNA gene sequence that most closely aligns with the complete 18S rRNA sequence of *C. glabrata* (accession no. X51831) in a BLAST search is that of *K. delphensis* (accession no. X83823), displaying just nine differences (0.5% divergence) when the complete (1798-bp) 18S sequences are aligned. Because of the limited number of complete 18S rRNA sequences deposited in the EMBL/GenBank database for these two organisms (one each) and their close similarity, the possibility that these four clinical isolates merely represent *C. glabrata* 18S sequence variants cannot be ruled out at this stage. Further work is being done to resolve this by determining the 18S sequence of a number of known *C. glabrata* and *K. delphensis* isolates, including those identified in this study, and in this manner establish whether *K. delphensis* is sometimes misidentified as *C. glabrata* in the routine laboratory.

The present study has demonstrated the application of RSCA as a high resolution typing tool capable of distinguishing sequence differences in a 281-bp region of the 18S rRNA gene sequence and its potential for identifying fungi of medical importance. While this analysis of the 281-bp region of the 18S rRNA gene sequence allowed a diverse range of fungal organisms to be identified, the discriminatory power of RSCA can be improved significantly by targeting a larger region of the 18S rRNA gene, containing greater sequence divergence. This would address some of the problems encountered in the present study, i.e., single nucleotide differences and identical sequences within the 281-bp target region. As the current typing system used a 979-bp and 940-bp region of the HLA-A and HLA-B genes respectively, for HLA targets, it should be possible to increase the region of the 18S sequence to the same order of magnitude, and this should allow the majority of medically important fungal species to be readily identified. Improved resolution was achieved with additional references in this study (data not shown) which parallels that already observed with HLA typing by RSCA [15–17]. Presently, three different FLRs are employed for HLA typing of the highly polymorphic HLA-B locus (in excess of 200 different allele sequences within the population). Therefore, it is envisaged that up to three FLRs will be needed to type all the important fungal organisms reliably and accurately. Such an approach will allow three RSCA migration values to be determined for each fungal sample, significantly improving the sensitivity and accuracy of the typing method and completely removing the ambiguities observed when a single FLR is used, as in the present study. An RSCA ladder of the common fungal pathogens similar to that employed for RSCA typing of the HLA genes can be generated easily, permitting immediate identification of the common pathogens from the gel image, and obviating the need to refer to a databank of fungal migration values

in these instances. In this study, mobility/migration values were assigned to the RSCA profiles generated for the fungi on the basis of arbitrary values of 1 and 1000 attributed to the fastest duplex which in all cases is the FLR homoduplex and the single-stranded FLR, respectively. As the migration of the RSCA profile of each fungal species relative to the two markers is constant for the electrophoresis conditions described, the scale allowed the correction of lane to lane variability, thus permitting the assignment of values representative of the mobility of individual fungal species and enabled subsequent comparison from one gel to another to be made reliably. Alternatively, it should be possible to include two appropriate mol. wt markers in each lane – one migrating at the bottom of the gel in front of the homoduplex FLR and the other at the top of the gel behind the single-stranded FLR – and to assign migration values to the RSCA profiles with respect to these markers. By constructing a large database of RSCA migration values for the common fungal pathogens with a range of different FLRs it should be possible, as demonstrated here, to identify rapidly any fungal species already within this database by RSCA and continuously build up the database by identifying fungal organisms that generate new RSCA migration values through nucleotide sequence analysis.

Although conventional culture techniques provide a reliable diagnosis of a fungal infection, delays of up to several weeks in identifying the organism dictate the use of standard empirical antifungal therapy rather than a more directed approach. This, together with the failure to detect fungal infections in all instances in fungal blood cultures, has provided the impetus for the development of new technologies, shifting the emphasis in recent years to the use of molecular approaches for the rapid detection and identification of fungal infections. While the most definitive molecular approach is nucleotide sequence analysis of the fungal 18S rRNA-derived PCR amplicons, such a strategy is not feasible at present for most routine laboratories, principally because of the expertise required, expense and most importantly the limited sample throughput. However, RSCA possesses the necessary attributes to overcome these above difficulties and establish itself as a molecular diagnostic technique that can be readily implemented into the routine setting. Furthermore, its potential for the detection of the particular fungal organisms present in a mixed infection, as supported by its use in HLA typing to distinguish the HLA alleles in the heterozygous setting and the marker ladder used in Fig. 4, makes it a particularly attractive methodology for further development. Furthermore, while the RSCA analysis was restricted to the single laser LI-COR 4200L in the present study, it is possible to perform this analysis on the ABI PRISM 3100/3700 capillary sequencers and, as a consequence, dramatically improve the throughput and remove the hands-on time required. A shift to these particular instruments would not only allow three different FLRs to be used

simultaneously for each sample by virtue of the use of different fluorescent labels but also permit mol. wt markers to be included for each sample for accurate determination of the RSCA migration values.

In conclusion, this study has shown the application of RSCA as a novel approach for the identification of a range of different fungal species and has demonstrated the potential of the technology for species confirmation of a small number of clinical isolates based on established RSCA profiles. This is particularly important for the *Candida* spp. which differ in their susceptibilities to antifungal drugs and the technique is ideally suited for the rapid identification of cultured fungi, facilitating a more selected, rather than an empirical, administration of antifungal treatment. Although this methodology is qualitative and, therefore, does not quantify fungal DNA load, it does possess the potential for quantification based on a competitive PCR approach, whereby a competitor 18S rRNA sequence, with a unique RSCA profile, is employed which can be differentiated from the fungal organism of interest and facilitate its quantification. It is possible that other medically relevant fungal species not tested in this study or without published 18S rRNA gene sequences may reveal patterns identical to those identified here. Studies are being done to address these issues and to assess the applicability of this approach to a range of clinical specimen types including blood samples for the detection of clinically important fungi, where this method could have a direct application in the routine laboratory.

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